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Fetal Germ Cells *in Vitro* and Its Inhibition by gp130-Mediated Signaling

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Mouse primordial germ cells (PGCs) arrive at the urogenital ridge (UGR) at around 10.5 days postcoitum (dpc). They proliferate until around 13.5 dpc, then enter into meiosis in the female or become mitotically arrested in the male gonads. In this study, meiotic transition of mouse PGCs was examined *in vitro*. Female PGCs obtained from UGRs or genital ridges at 10.5–11.5 dpc began to express meiosis-specific genes, *Scp3* and *Dmc1*, after dissociation and cultivation on feeder cells for several days. Meiotic transition into the leptotene stage was confirmed by the formation of axial cores. Male PGCs at 10.5–11.5 dpc and migratory PGCs obtained from mesenteries at 10.5 dpc also expressed Scp3 and formed axial cores after several days of culture, supporting the hypothesis that PGCs are capable of entering meiosis before arriving at the UGR. gp130-mediated signaling, known to promote survival/growth of PGCs and also to inhibit the differentiation of embryonic stem cells, suppressed the expression of Scp3 in PGCs and inhibited the following formation of axial cores *in vitro*. This novel activity of gp130-mediated signaling may provide some clues for the understanding of pluripotency of mammalian germ-line cells and/or the sex differentiation of fetal germ cells. © 2000 Academic Press

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INTRODUCTION

Mouse primordial germ cells (PGCs), which are derived from a proximal population of epiblast cells, are first recognizable in the posterior region of the extraembryonic mesoderm by their surface alkaline phosphatase (ALPase) activity at around 7.25 days postcoitum (dpc) (Chiquoine *et al.*, 1954; Ginsburg *et al.*, 1990; Tam and Zhou, 1996). Then, they proliferate rapidly and migrate to reach the urogenital ridge (UGR) at around 10.5 dpc. PGCs continue to divide mitotically several additional times and take different fates according to the sex of embryos at around 13.5 dpc. They enter into the prophase of the first meiotic division in the female, while those in the male gonads become mitotically arrested as prospermatogonia (Hilscher *et al.*, 1974). Previous studies have indicated that the sex differentiation of the germ cells is directed by the sex of the gonadal somatic cells and not by that of the germ cells themselves. Germ cells resume mitosis as spermatogenic stem cells or they progress in meiosis as oocytes after birth (reviewed in McLaren, 1994).

Proliferation and migration of mouse PGCs have been studied *in vitro*, in addition to the analyses of a few genetic mutants (reviewed in Wylie, 1999). Mouse PGCs can be grown for several days on suitable feeder cell layers, showing a limited capacity for proliferation that correlates with their cessation of cell division *in vivo* (Donovan *et al.*, 1986; Ohkubo *et al.*, 1996). Previous studies using such *in vitro* culture systems identified several growth/survival-promoting factors for PGCs. Steel factor (SLF), encoded by the *Steel* loci, was shown to be essential for the survival of PGCs *in vitro* as well as *in vivo* (Dolci *et al.*, 1991; Godin *et al.*, 1991). Leukemia inhibitory factor (LIF), another survival-promoting factor of PGCs, promotes proliferation of PGCs in combination with SLF *in vitro* (De Felici *et al.*,

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1991; Matsui *et al.*, 1991). The LIF-specific receptor component, the low-affinity LIF receptor, is expressed on the cell surface of PGCs (Cheng *et al.*, 1994). LIF belongs to the interleukin-6 (IL-6) cytokine family that uses gp130 as a common signal-transducing subunit of the receptor complexes (Saito *et al.*, 1992). Recently, oncostatin M (OSM), another member of the IL-6 cytokine family, was shown to promote the growth and/or survival of PGCs *in vitro* (Hara *et al.*, 1998). gp130-mediated signaling also exerts a well-characterized differentiation-inhibitory activity on pluripotential embryonic stem (ES) cells (Smith *et al.*, 1988; Williams *et al.*, 1988) and is also essential for the derivation of pluripotential embryonic germ (EG) cells from PGCs *in vitro* (Matsui *et al.*, 1992; Resnick *et al.*, 1992; Koshimizu *et al.*, 1996).

Cytological studies of nuclear morphology and chromosomal preparations of fetal germ cells indicated that female PGCs cease mitosis and enter into the prophase of the first meiotic division at around 13.5 dpc, progressing through the preleptotene, leptotene, zygotene, pachytene, and diplotene stages and to diakinesis before arresting after birth (Borum, 1961; Speed, 1982). At the leptotene stage, each pair of sister chromatids forms a meiosis-specific longitudinal axial core to which the chromatin loops are attached. The Scp3 (Cor1/Sycp3) protein is one of the components of these axial cores (Heyting *et al.*, 1988; Dobson *et al.*, 1994; Klink *et al.*, 1997). Scp3 is a good marker to detect the meiotic transition, since its expression is specific for meiosis and it functions from the initial step of the first meiotic division (Yuan *et al.*, 2000). At the zygotene stage, the axial cores of each pair of homologous chromosomes become aligned in parallel, thereby becoming the lateral elements of the synaptonemal complexes, the structure considered to be involved in the chromosome condensation, pairing, recombination, and segregation. The Dmc1 protein is a meiosis-specific mammalian RecA homologue that is supposed to function during chromosome synapsis and homologous recombination events (Sato *et al.*, 1995; Pittman *et al.*, 1998; Yoshida *et al.*, 1998).

Compared with studies of growth regulation, however, relatively little is known about the developmental aspects of sex differentiation of PGCs into oocytes or prospermatogonia in fetal gonads. Observation of ectopic germ cells in the mesonephroi or adrenal cortex (Zamboni and Upadhyay, 1983; Francavilla and Zamboni, 1985) suggested that both male and female PGCs that had arrived within the vicinity of the gonads, or related environments, showed cell-autonomous entry into meiosis if they were set apart from the male gonadal environments. Studies of reaggregated cultures of embryonic gonads (McLaren and Southee, 1997) also demonstrated that both female and male PGCs at 10.5–11.5 dpc, shortly after arriving at UGRs, entered into meiosis when they were surrounded by lung somatic cells. However, precise examination of the meiotic transition and identification of its regulative factors have been impeded, possibly due to the lack of a dispersed culture system for PGCs, in which the meiotic transition could be detected and analyzed in detail.

In this study, we first showed that female PGCs obtained from UGRs or genital ridges at 10.5–11.5 dpc, at the stages when PGCs were still dividing mitotically, began to express Scp3 (Cor1/Sycp3) and Dmc1, followed by the formation of axial cores after dissociation and cultivation on feeder cells for several days. Migratory PGCs obtained from mesenteries at 10.5 dpc and male PGCs from 10.5 to 11.5 dpc also entered into the leptotene stage after culturing, thus supporting the hypothesis that PGCs of both sexes are capable of entering meiosis before arriving at the UGR. We next demonstrated that gp130-mediated signaling suppressed the meiotic transition of female PGCs *in vitro*. This novel activity of gp130-mediated signaling may provide some clues for the understanding of pluripotency of mammalian germ-line cells and/or the factors involved in the sex differentiation of fetal germ cells.

MATERIALS AND METHODS

Mice

Random-bred Jcl:ICR mice were purchased from CLEA (Shizuoka, Japan) and maintained in a controlled environment with light/dark cycles of 8:00 AM to 8:00 PM. The day when a copulation plug was found was designated 0.5 dpc. UGRs at 10.5 dpc, genital ridges at 11.5 dpc, or gonads after 12.5 dpc were dissected out to obtain gonadal PGCs. Migratory PGCs, before their arrival at the UGRs, were obtained by dissecting out dorsal mesenteries that were devoid of UGRs at 10.5 dpc.

Determination of Sex by Ube1 Genotyping

PCR primers were designed based on *Ube1X* sequence (Imai *et al.*, 1992): 5′-TGGTCTGGACCCAAACGCTGTCCACA-3′ and 5′-GGCAGCAGCCATCACATAATCCAGATG-3′.

Pieces of embryonic tissues that had been boiled in water and mixed by vortexing were directly used for PCR templates. Amplification was performed using LA *Taq* (Takara) at 94°C for 1 min and 30–35 cycles of 98°C for 15 s, 66°C for 20 s, followed by an elongation step of 1 min at 72°C. Mg^{2+} and dNTP concentrations were 2 and 0.4 mM, respectively. PCR products were electrophoresed on 2% agarose gels. Primers for *Sry* (Gubbay *et al.*, 1990) were 5′-CTGTGTAGGATCTTCAATCTCT-3′ and 5′-GTGGTGAGAGGCACAAGTTGGC-3′ (kindly provided by Dr. K. Morohashi).

RT-PCR Analyses

Solid phase RT-PCR was performed by mRNA purification using Dynabeads oligo$(dT)_{25}$ (Dynal), followed by reverse transcription using Superscript II (GIBCO), which produced cDNAs directly attached to the particles. PCR primers were as follows: *Scp3*, 5′-ATGATGGAAACTCAGCAGCAAGAGA-3′ and 5′-TTGACACAATCGTGGAGAGAACAAC-3′; *Dmc1*, 5′-GGACATTGCTGACCGCTTCAACGT-3′ and 5′-GGCGATCCTCAGTTCTCCTCTTCC-3′; *Oct3/4*, 5′-ATTCTCGAACCTGGCTAAGCT-3′ and 5′-ATGGTGGTCTGGCTGAACACCTTT-3′; and *GAPDH*,

5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′. Primers for *LIF* were 5′-CCTCTTCCCATCACCCCTGTAAAT-3′ and 5′-ACTTGGTCTTCTCTGTCCCGTTGC-3′, which amplified both the matrix-associated and the diffusible forms of *LIF* (Rathjen *et al.*, 1990).

Each pair of primers was designed from sequences in separate exons. Specific amplification with each pair of primers was confirmed by direct sequencing of the RT-PCR products of 13.5-dpc male and female gonads. The cycle numbers for each primer set were determined by electrophoresis of the PCR products at several points of amplification cycles preliminarily.

Real-time PCR was carried out for relative quantification of the expression of *Scp3*, *Dmc1*, and *LIF* by using SYBR Green PCR and RT-PCR reagents and the ABI Prism 7700 sequence detection system (PE Biosystems). One hundred nanograms of total RNAs was reverse transcribed by using Superscript II and random hexamers as primers and was used as template. Specific amplification was confirmed by electrophoresis and direct sequencing of the PCR products. Reverse transcriptase-negative controls were included for each series of reactions. *GAPDH* was used as an internal standard. ΔC_t value is the difference in the threshold cycles between the target gene (*Scp3*, *Dmc1*, or *LIF*) and the internal standard (*GAPDH*). Assays were run in triplicate and mean values are presented.

The oligonucleotide probes for murine *LIF* and *GAPDH* were 5′-GAAAAGCTATGTGCGCCTAA-3′ and 5′-TGCTGAGTATGTCGTGGAGT-3′, respectively. Southern blotting of PCR products and subsequent hybridization were performed following standard procedures. The probes were end-labeled with [^{32}P]ATP and signals were visualized using a BAStation 2000 system (Fuji). The template for an exogenous control RNA was made by deleting 41 bp from a 234-bp PCR product of endogenous *LIF*. A T7 promoter sequence and oligo(dT)$_{25}$ were attached to each end of the fragment, and RNA transcripts were made using T7 RNA polymerase (Promega). Reverse transcription was performed using Superscript II and random hexamers and oligo(dT)$_{12–18}$ as primers.

Preparation of Anti-Scp3 Antibody

A synthetic oligopeptide (PPLVDQPKKAFDFEKDDKDC) based on the amino acid sequence near the N-terminal of the mouse Scp3 (Cor1/Sycp3) protein (Heyting *et al.*, 1988; Dobson *et al.*, 1994; Klink *et al.*, 1997) was conjugated to keyhole limpet hemocyanin as a carrier protein, mixed with adjuvant, and used to inoculate rabbits four times (carried out by Sawady, Japan). Western blotting was performed using PVDF membranes (Millipore) following standard procedures. Affinity-purified antibody showed no apparent difference in immunostaining signals compared to unpurified rabbit antiserum. Therefore, we used unprocessed antiserum for routine immunostaining.

Histology and Immunostaining

Tissues for frozen sections were embedded in OCT compound (Tissue-Tek) and cut at 10 μm thickness. The spreading procedure of testicular or cultured cells was carried out by using 0.1 M sucrose for hypotonic treatment and 1% paraformaldehyde and 0.1% Triton X-100 for the spreading/fixation solution, as described by Peters *et al.* (1997). Sections, spread samples, and cultured cells were fixed with 4% paraformaldehyde in PBS− (Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline) for 15 min, permeabilized with acetone for 15 min, and blocked for 1 h with 3% bovine serum albumin (BSA) and 10% normal goat serum in PBS−. The anti-Scp3 immune serum was diluted to 1/500 with 0.1% BSA in PBS− and used for incubation for 2 h at room temperature or overnight at 4°C. Tetramethylrhodamine-conjugated goat anti-rabbit Igs (Biosource) were used for the second antibody. Anti-Dmc1 staining was performed as described (Yoshida *et al.*, 1998). Anti-SSEA-1 and ALPase staining was carried out as described previously (Donovan *et al.*, 1986). In some experiments, nuclei were counterstained with Hoechst 33258 dye. Microscopic images were obtained using a cooled CCD camera mounted on a fluorescence microscope (Photometrics) or by a confocal microscope (Carl Zeiss).

Cell Culture

Embryos were stored in a 1:1 mixture of the culture medium and PBS− at 4°C during the procedure of sex determination, then the tissues containing PGCs were dissected out. After the trypsin digestion and filtration, single-cell suspensions were plated onto mitomycin C-treated feeder cells that produce the membrane-bound form of SLF (Sl/Sl4 m220; kindly provided by Dr. Y. Matsui) (Zsebo *et al.*, 1990). The feeder cells had been prepared in chamber slides (Nunc) approximately 24 h before the onset of primary culture. The culture medium was Dulbecco's modified Eagle's medium (with high glucose and HEPES and without phenol red; GIBCO 21063-029), supplemented with 1 mM sodium pyruvate; 20% fetal calf serum; 1 mM nonessential amino acids; 30 μM adenosine, guanosine, cytidine, and uridine; 10 μM thymidine; 100 μM 2-mercaptoethanol; and penicillin and streptomycin. Culture samples were incubated at 37°C with 5% CO_2.

Cytokines and Additives

Recombinant mouse LIF and OSM were purchased from GIBCO and R&D Systems, respectively. Recombinant IL-6 and an extracellular soluble form of recombinant IL-6 receptor were provided by Dr. T. Taga (Tokyo Medical and Dental University, Japan).

RESULTS

Determination of the Sex of Mouse Embryos by Ube1 Genotyping

We used genomic PCR of mouse *Ube1* genes in order to determine the sex of mouse embryos. There are two *Ube1* gene members in mice, *Ube1X* on the X chromosome and *Ube1Y* on the Y chromosome (Imai *et al.*, 1992). A pair of primers was designed that could amplify fragments of both *Ube1X* and *Y*, but would result in different lengths of amplification products, due to several deleted regions between the two genes. Thus, two distinct bands were amplified from male samples and a single band from female samples (Fig. 1). The advantage of using these genes for sex determination is that *Ube1X* serves as an amplification control in each reaction.

Expression of Meiosis-Specific Genes in Fetal Germ Cells

We first carried out solid-phase RT-PCR to detect the expression of the *Scp3* and *Dmc1* genes in both male and

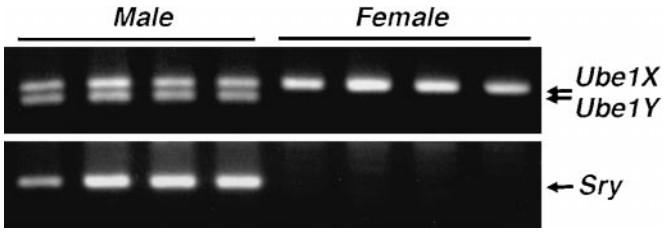

FIG. 1. Determination of the sex of mouse embryos by *Ube1* genotyping. Top: Tissue fragments of embryos at 11.5 dpc were boiled in water and directly used as templates for genomic PCR of the *Ube1* genes. Male samples gave two distinct bands of 217 and 198 bp, while the female product was a single band of 217 bp. Bottom: Genomic PCR for *Sry*.

female genital ridges and gonads at 11.5–13.5 dpc. In the female, amplification products from both *Scp3* and *Dmc1* were detected from 12.5 dpc. Unexpectedly, amplification products from male gonads were also detected at 12.5–13.5 dpc (Fig. 2A). Real-time PCR of *Scp3* and *Dmc1* showed that differences in the expression between male and female gonads at 13.5 dpc were about 8- and 32-fold, respectively (mean values of ΔC_t (difference in the threshold cycles between *Scp3* or *Dmc1* and *GAPDH*) for male and female gonads, 10.2 and 7.1 for *Scp3,* 9.2 and 4.2 for *Dmc1*).

We next examined the expression of both genes by female PGCs after cultivation on feeder cells. Female UGRs at 10.5 dpc were dissociated and cultured on Sl/Sl4 m220 feeder cells for 1, 3, or 5 days. RT-PCR products from both *Scp3* and *Dmc1* increased after 3 days of culture. The relative increases in the expression of *Scp3* and *Dmc1* between day 1 and day 3 of the culture were about three- and six-fold, respectively, when estimated by real-time PCR (ΔC_t values for days 1 and 3, 18.4 and 16.8 for *Scp3,* 18.6 and 16.0 for *Dmc1*). In contrast, the product of the *Oct3/4* gene decreased during the same period of culture (Figs. 2A and 2B). *Oct3/4* is expressed only in embryonic and germ-line stem cells, and its expression declines in female germ cells at around 13.5 dpc *in vivo* (Pesce *et al.,* 1998). Since the number of the germ cells between day 1 and day 3 of the culture did not substantially change (Fig. 5A), it was suggested that female PGCs increased the expression of *Scp3* and *Dmc1* after dissociation and cultivation on feeder cells.

Detection of the Meiotic Transition in Vitro by Anti-Scp3 Immunostaining

The axial core is a characteristic and highly specific structure of the meiotic division. We raised a rabbit polyclonal antibody against a synthetic polypeptide from the amino acid sequence of the mouse Scp3 protein (Klink *et al.,* 1997), in order to detect the meiotic transition by examining its expression and localization in fetal germ cells both *in vivo* and *in vitro*. Immunoblotting of adult brain, heart, liver, and testis with the antiserum detected the expected band of approximately 30 kDa only in the testis (data not shown). Axial cores were clearly detected in spermatocytes by immunostaining of frozen sections and

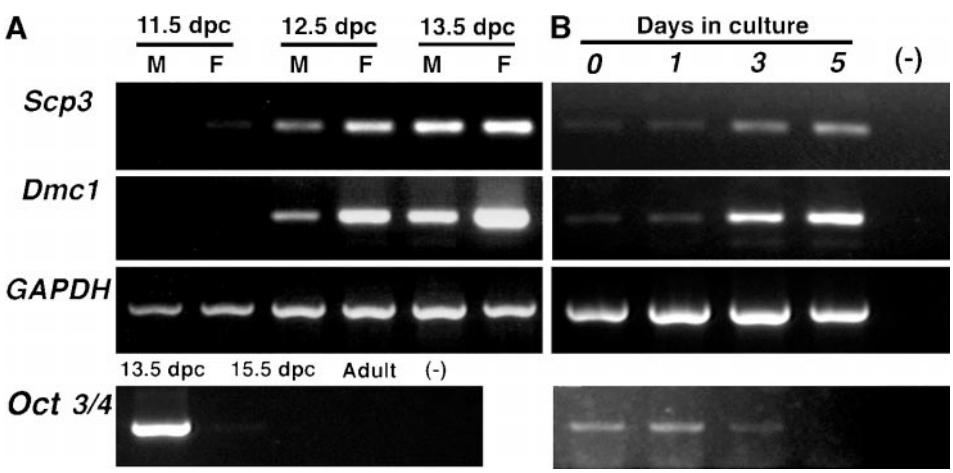

FIG. 2. Expression of meiosis-specific genes *in vivo* and *in vitro*, analyzed by RT-PCR. (A) Upper three rows: Solid-phase cDNAs were made from the equivalent of one genital ridge at 11.5 dpc or one gonad at 12.5 or 13.5 dpc. The same templates were used sequentially for RT-PCR of three genes. M, male; F, female. Cycle numbers for *Scp3, Dmc1,* and *GAPDH* were 25, 25, and 20, respectively, and were set to indicate the appearance of male products at 12.5 dpc. The lowest row: RT-PCR of female gonads at 13.5 and 15.5 dpc and adult ovary for *Oct3/4.* 100 ng of total RNAs was used as template. Cycle number was 25. (B) Equivalents of one female UGR at 10.5 dpc were dissociated and plated onto 3×10^5 Sl/Sl4 m220 feeder cells in each culture well. The samples were collected at 1, 3, or 5 days after culture and processed for the solid-phase RT-PCR. (−) indicates a control without reverse transcriptase. Cycle numbers for *Scp3, Dmc1, GAPDH,* and *Oct3/4* were 30, 30, 25, and 30, respectively.

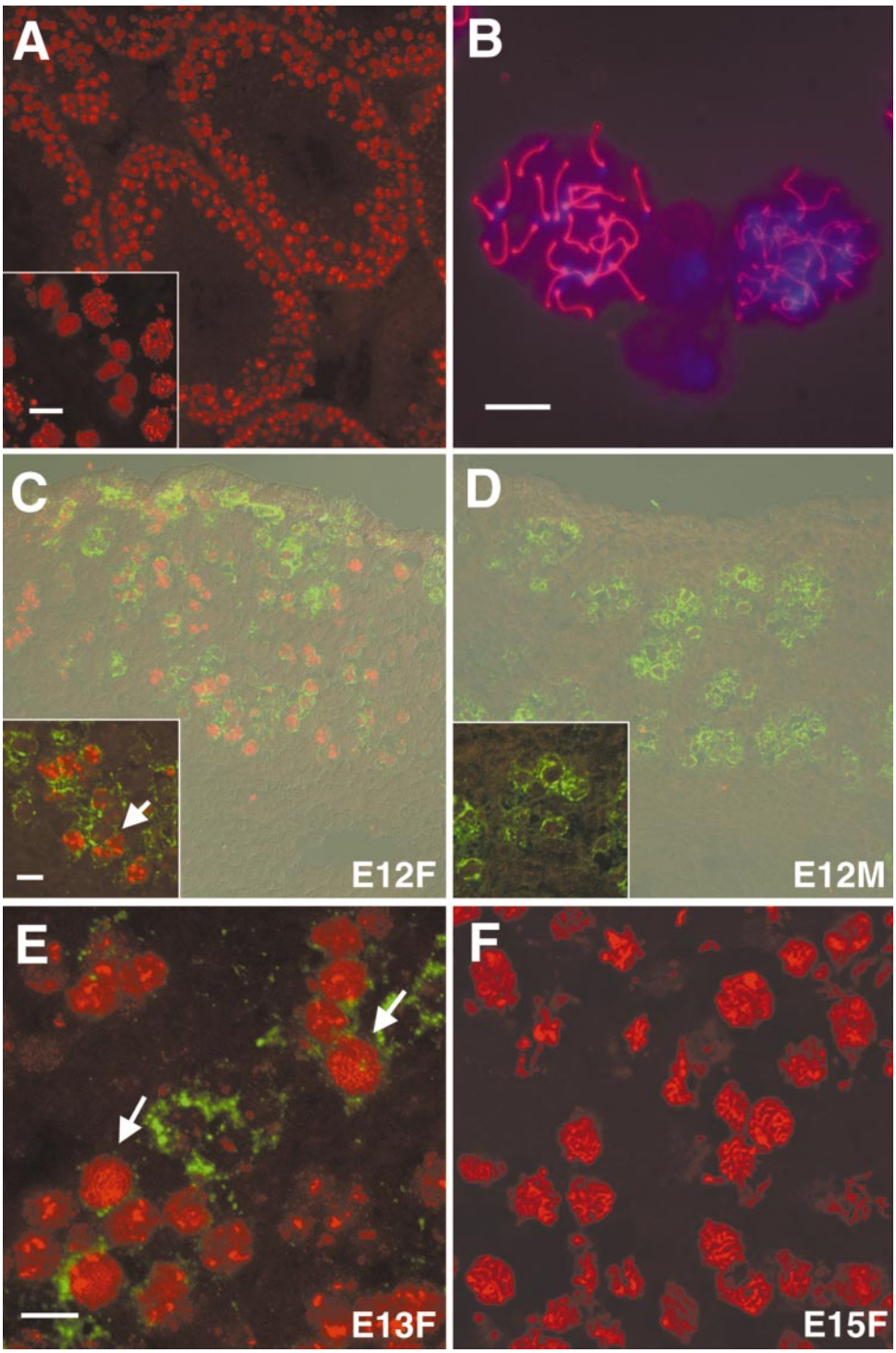

FIG. 3. Meiotic transition *in vivo*, examined by immunostaining with anti-Scp3 antiserum (A–F, red) and anti-SSEA-1 antibody (green, C–E). (A) A frozen section of an adult testis. (B) Spread samples of adult testicular cells, counterstained with Hoechst 33258 dye. The left nucleus shows pachytene appearance. The bivalents are forming synapses in the right nucleus. (C) In a 12.5-dpc female gonad, germ cells identified by SSEA-1 express Scp3 with characteristic foci inside the nuclei (arrow). The fluorescent image was overlaid onto the Nomarski image. The inset shows higher magnification view of the fluorescent signals. (D) At 12.5 dpc, male germ cells do not show detectable Scp3 signals. (E) At 13.5 dpc in the female, axial cores are observed as fine fibrous alignment of Scp3 signals (arrows). (F) At 15.5 dpc in the female, the Scp3 signals become thicker, which corresponds to the zygotene–pachytene stages. Scale bar: 10 μm. (C, D) and (E, F) are at the same magnifications, respectively.

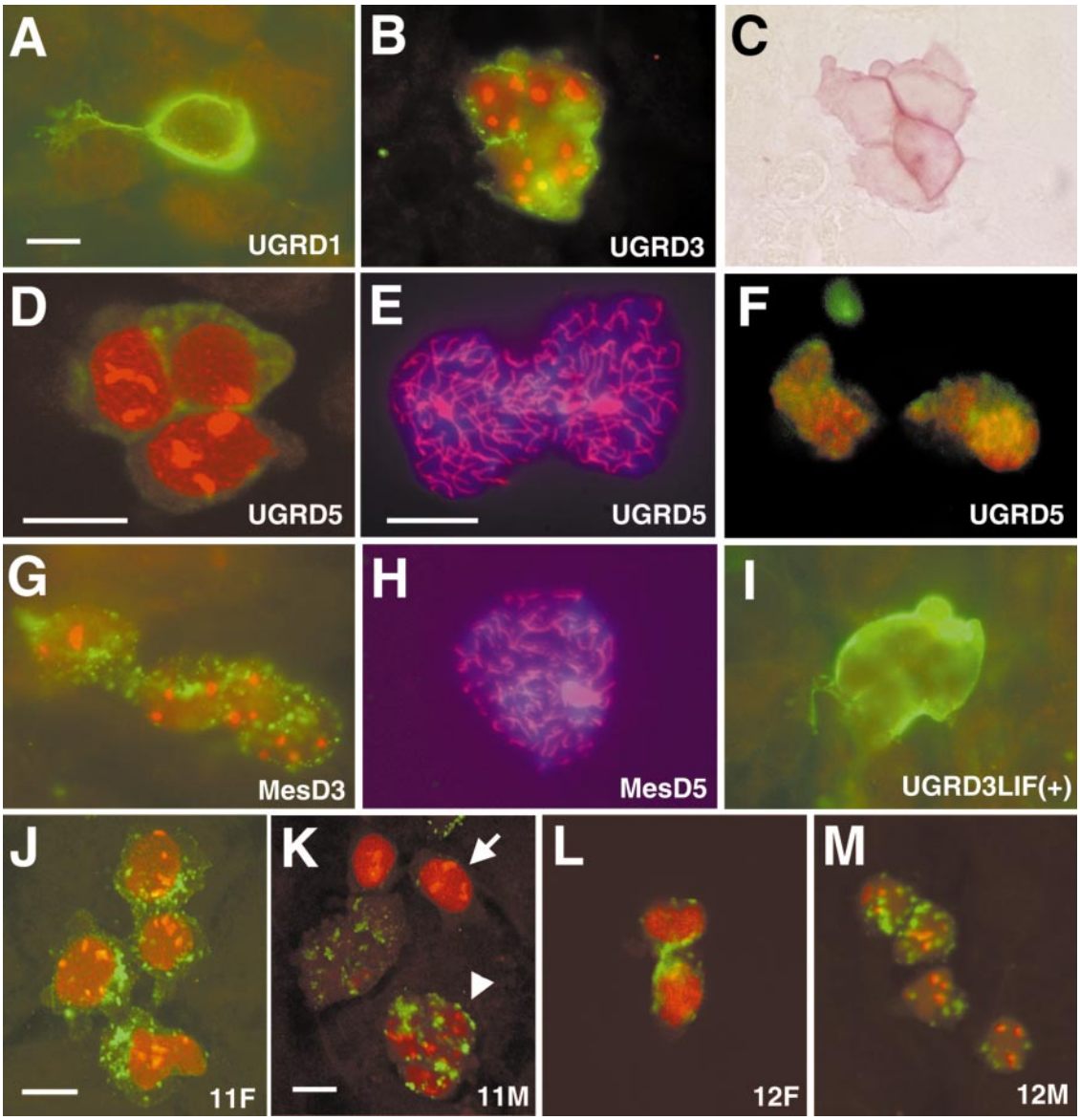

FIG. 4. Meiotic transition of PGCs *in vitro.* UGRs (A–F, I) or mesenteries (G, H) at 10.5 dpc, genital ridges at 11.5 dpc (J, K), or gonads at 12.5 dpc (L, M) were dissociated and cultured on the Sl/Sl4 m220 feeder cells. Immunostaining with anti-Scp3 (red, A, B, D, E, and G–M), anti-Dmc1 (red, F), and anti-SSEA-1 (green, A, B, D, F, G, and I–M). (A, B) Female UGRs at 10.5 dpc were cultured for 1 (A) or 3 (B) days. The germ cells identified by SSEA-1 show the Scp3 signals at day 3 (B). (C) A bright-field view of (B), stained for ALPase activity (red). (D) Female UGRs at 10.5 dpc cultured for 5 days. Axial cores are observed as fine fibrous alignment of the Scp3 signals. (E) Spreading of a sample similar to that in (D). Axial cores are clearly seen. Nucleus was counterstained with Hoechst 33258 dye. (F) Female UGRs at 10.5 dpc cultured for 5 days, stained with anti-Dmc1 and SSEA-1. (G, H) Female mesenteries at 10.5 dpc cultured for 3 (G) or 5 days (H, spread sample). (I) 10^3 units/ml LIF was added to the culture of female UGRs at 10.5 dpc for 3 days. Scp3 signal is not detectable. (J, K) Female (J) and male (K) genital ridges at 11.5 dpc cultured for 3 days. The formation of axial cores is observed both in (J) and in (K, arrow). In (K), the cells with weak Scp3 staining (arrowhead) are also observed. (L, M) 12.5-dpc female (L) or male (M) gonads cultured for 3 days. The male germ cells show much weaker signals of Scp3 than the females. Scale bar: 10 μm. (A–C, F, G, I), (E, H), and (K–M) are at the same magnifications, respectively.

spread samples of adult testes (Figs. 3A and 3B). Somatic cells examined did not show any significant staining with this antiserum. Staining with preimmune serum also did not show any significant signal in both germ and somatic cells (data not shown). The specificity of this antiserum was almost consistent with those of the anti-rat or anti-golden

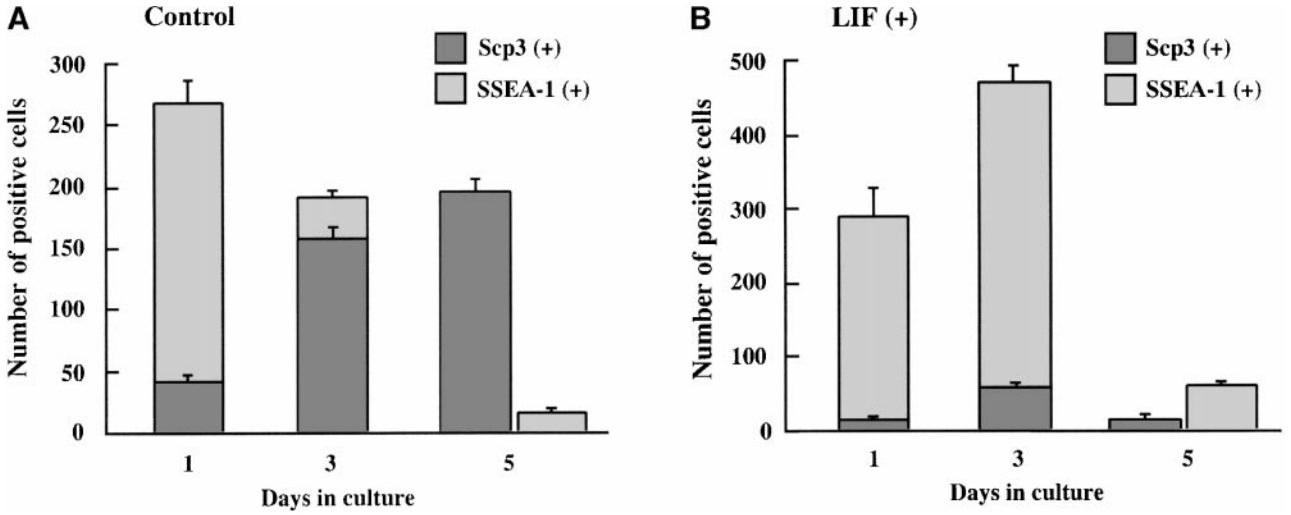

FIG. 5. Changes in the number of SSEA-1- and/or Scp3-positive cells *in vitro.* The equivalent of half of one female UGR at 10.5 dpc was dissociated and plated onto feeder cells in each culture well. After 1 or 3 days of culture, almost all the Scp3-positive cells expressed SSEA-1 signals. After 5 days, SSEA-1 signals on Scp3-positive cells became weak or undetectable, thus the numbers are shown separately. (A) Control without LIF. (B) 10^3 units/ml LIF was added to the culture medium. Means and SEM from four duplicate wells are indicated.

hamster Cor1 (Scp3) antibodies used in the previous studies (Heyting *et al.*, 1988; Dobson *et al.*, 1994).

We next examined the presence and localization of the Scp3 protein in embryonic gonads at 11.5–15.5 dpc by using this antiserum. In female germ cells, Scp3 was detected inside the nuclei with intense dot-like signals at 12.5 dpc (Fig. 3C). In male germ cells, no detectable signal was observed at 12.5 dpc (Fig. 3D). At 13.5 dpc in the female, formation of axial cores became visible as fine fibrous structures inside nuclei, in addition to the remaining dot-like staining (Fig. 3E). The fibers inside nuclei became thicker at 15.5 dpc in the female, corresponding to the progression of meiosis to zygotene–pachytene stages (Fig. 3F). Unexpectedly, testicular tubules in the testis at later stages also contained several cells that showed characteristic dot-like staining inside nuclei, although the signals were very weak. The dot-like pattern of the Scp3 signals showed no discernible correlation with the distribution of DNA stained with Hoechst 33258 dye (data not shown). It may represent self-assembly of the protein as was reported for the rat Scp3 protein overexpressed in cultured cell lines (Yuan *et al.*, 1998).

We next examined whether female PGCs start to express the Scp3 protein and to form axial cores after culture on feeder cells. Female genital ridges at 10.5 dpc were dissociated and cultured on Sl/Sl4 m220 feeder cells for 1, 3, or 5 days, then fixed and stained for SSEA-1, ALPase, and Scp3. After 1 day, PGCs were scattered on feeder cells with strong SSEA-1 (Fig. 4A) and ALPase signals. A few PGCs exhibited weak staining for Scp3. After 3 days of culture, PGCs formed cell colonies that were labeled with reduced levels of SSEA-1 and ALPase activity. Coinciding with the reduction of these surface markers, many of the colonies exhibited staining for Scp3 with characteristic dot-like signals inside the nuclei (Figs. 4B and 4C).

After 5 days of culture, intensity of the Scp3 signals increased, and there also appeared cells that formed axial core structures (Fig. 4D). Formation of axial cores was further examined with the spreading procedure of the cultured cells by hypotonic treatment (Fig. 4E). This axial core formation was comparable to that observed in female germ cells at around 13.5 dpc *in vivo* and thus confirmed that female PGCs entered into the leptotene stage of the first meiotic division *in vitro.* We also observed germ cells that expressed the Dmc1 protein, providing further evidence for the meiotic transition *in vitro* (Fig. 4F).

Changes in the number of SSEA-1- and/or Scp3-positive cells are shown in Fig. 5A. Intensity of the Scp3 staining varied among the cells, and the progression of axial core formation was also gradual and variable. Interestingly, however, cells in the same cluster tended to show similar intensities of the Scp3 signal or similar progressions of the axial core formation (Fig. 4K). This may imply a degree of synchrony in the meiotic transition in the same cell colony, which may be related to the observation that female PGCs form cysts and show synchrony in the mitotic divisions *in vivo* (Pepling *et al.*, 1998).

We next cultured migratory PGCs obtained from mesenteries at 10.5 dpc, in order to examine whether PGCs are capable of entering into meiosis without reaching the UGRs. Again, migratory PGCs started to express Scp3 after culture for several days. There also appeared cells that formed axial cores, as was observed in the culture of PGCs obtained from UGRs (Figs. 4G and 4H).

TABLE 1
Effects of LIF and OSM on the Number of Scp3 (+) Cells after Culture of Fetal Germ Cells

Stage of sample	No. of Scp3 (+) cells (%)		
	Control	LIF	OSM
11.5 dpc	296 ± 28 (100%)	61 ± 8 (21)	117 ± 13 (40)
12.5 dpc	694 ± 35 (100)	492 ± 43 (71)	526 ± 35 (76)
13.5 dpc	698 ± 37 (100)	711 ± 46 (102)	747 ± 39 (107)

Note. The equivalent of one-half, one-third, or one-fourth of a female genital ridge or gonad at each stage was dissociated and cultured on the Sl/Sl4 m220 feeder cells in each culture well for 3 days. Data are represented as means and SEM of the numbers of Scp3-positive cells from four duplicate wells. Parentheses, percentage change of the mean value at each stage with control designated as 100%. Additives and concentrations: LIF, 10^3 units/ml; OSM, 25 ng/ml.

Male PGCs obtained from genital ridges or UGRs at 10.5–11.5 dpc also started to express Scp3 and began to form axial cores after several days of culture in a manner similar to that of female PGCs obtained from the same stages (Figs. 4J and 4K). On the other hand, male germ cells obtained from developing testis at 12.5 or 13.5 dpc showed dot-like staining of Scp3, but there were only few cells that had formed axial cores. These Scp3 signals were stronger than those observed in the germ cells in male embryonic gonads at the corresponding stages, but they were much weaker than those observed in the female germ cells cultured from 12.5 to 13.5 dpc (Figs. 4L and 4M) or than those in both female and male PGCs cultured from 10.5 to 11.5 dpc.

Effects of gp130-Mediated Signaling on the Meiotic Transition of Female PGCs

We next examined the effects of several growth factors (LIF, 10^3 units/ml; bFGF, 10 ng/ml; forskolin, 10 μM; retinoic acid, 10 μM) on the expression of Scp3 in PGCs *in vitro*. Among them, LIF markedly suppressed the expression of Scp3 in PGCs. Addition of 10^3 units/ml of LIF to the culture of female UGRs at 10.5 dpc increased the number of PGCs as previously reported, but both the expression of Scp3 and the following formation of axial cores in the PGCs were inhibited strongly compared to that of the control culture (Table 1, Figs. 4I, 5A, and 5B). The proliferation/survival-promoting activity and this suppressive effect on the Scp3 expression by LIF showed similar dose dependency (Fig. 6A). Oncostatin M, another member of the IL-6 cytokine family known to promote the proliferation/survival of PGCs, also reduced the number of Scp3-positive cells (Table 1). A complex of IL-6 and soluble IL-6 receptor (sIL-6R), which binds to cell surface gp130 and directly activates intracellular signaling (Taga *et al.*, 1989), showed effects similar to those of LIF and OSM (Fig. 6B). Since such reduction in the number of Scp3-positive cells by the ligands became smaller as the stages of the gonads progressed (Table 1), these factors probably affect the transition into meiosis, rather than after the transition.

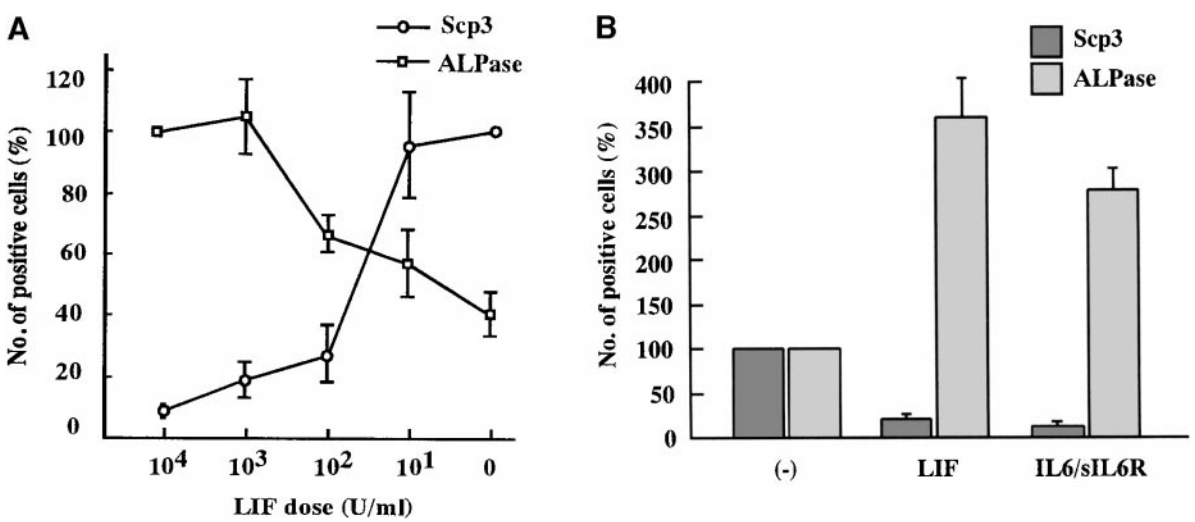

FIG. 6. Dose effects of LIF (A) and effects of LIF and the IL-6/sIL-6R complex (B) on the number (%) of ALPase- and Scp3-positive cells *in vitro*. The equivalent of half of one genital ridge at 11.5 dpc was dissociated and cultured on feeder cells in each culture well for 3 days. 10^3 units/ml LIF or 150 ng/ml IL-6 and 500 ng/ml sIL-6R were added to the culture medium in (B). Means and SEM from six duplicate wells are indicated.

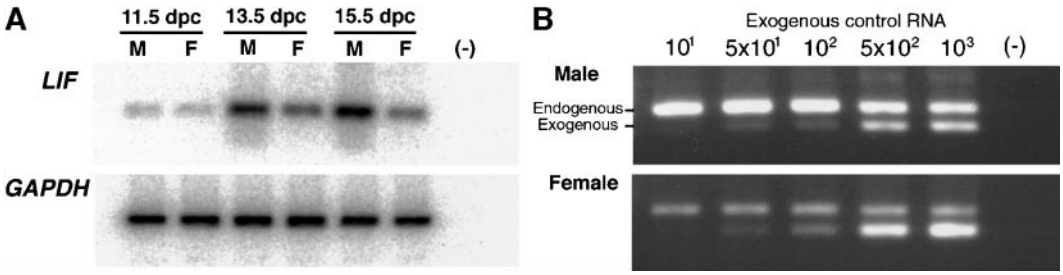

FIG. 7. Differential expression of *LIF* in embryonic gonads. (A) Solid-phase RT-PCR for *LIF* and *GAPDH*. cDNAs were made from the equivalent of one genital ridge at 11.5 dpc, one-half gonad at 13.5 dpc, or one-third gonad at 15.5 dpc. The same templates were used for the amplification of both genes and the products were blotted and hybridized with oligonucleotide probes for each gene. Cycle numbers for *LIF* and *GAPDH* were 35 and 25. (B) RT-PCR for *LIF* of male and female gonads at 13.5 dpc in the presence of exogenous control RNAs. Half-log dilutions of the control RNAs were added to the RT reaction containing 200 ng of total RNAs. The upper band in each lane represents the amplification product from endogenous *LIF* and the lower band is derived from the exogenous control. Cycle number was 35. (−) indicates a control without reverse transcriptase containing 200 ng of total RNA of male gonads and 10^3 molecules of the exogenous control RNAs.

Differential Expression of LIF in Embryonic Gonads

If LIF or OSM is related to the factors that prevent the meiotic transition of PGCs in the male gonad, their expression should be higher in the male than in the female gonads. Alternatively, it should be low enough in both sexes so that it shall not interfere with the sex differentiation of fetal germ cells. Since the expression of *LIF* in embryonic gonads was not detected in *in situ* hybridization study, and since previous studies also reported that the signals of *LIF* were very weak in RNase protection assays (Robertson *et al.*, 1993), we used RT-PCR methods to detect the expression of *LIF* in embryonic gonads. Amplification products of *LIF* were detected both in male and in female gonads, but the signals in male gonads became stronger than in the female during 11.5–13.5 dpc (Fig. 7A). RT-PCR analysis in the presence of exogenous control RNAs showed approximately 5-fold difference in the *LIF* expression between male and female gonads at 13.5 dpc (Fig. 7B). Real-time PCR of *LIF* further confirmed that the relative difference at 13.5 dpc was about 4.3-fold (ΔC_t values for male and female gonads were 11.9 and 14.0). The expression of *OSM* in fetal gonads was reported previously (Hara *et al.*, 1998). However, we detected only very small amount of *OSM* by RT-PCR both in male and in female gonads, although several combinations of primers for *OSM* were examined.

DISCUSSION

Autonomous Transition into Meiosis of Mouse PGCs

We tried to devise a dispersed culture system of PGCs in which patterns of meiotic transition and its regulative factors could be examined more precisely than in the previous organ or reaggregate culture systems. By using meiosis-specific markers, we found that PGCs started to express the meiosis-specific genes and to form axial cores after dissociation and cultivation on feeder cells. Since SSEA-1 and ALPase signals decreased during the same period of culture, previous observations of autonomous disappearance of PGCs cultured on feeder cells seem to be, at least in part, a consequence of the reduction of PGC surface markers and not the disappearance of the cells themselves.

We showed that PGCs isolated from the mesenteries at 10.5 dpc, prior to their arrival at and probably before being influenced by the UGRs, were capable of expressing Scp3 and forming axial cores after cultivation for several days. Previous observations on ectopic oocytes in the adrenal cortex or mesonephroi revealed that meiosis could occur outside the ovary (Zamboni and Upadhyay, 1983; Francavilla and Zamboni, 1985). However, it is possible that these ectopic oocytes are still influenced by gonad-related environments, because the mesonephroi is closely related to the gonads and also because the adrenal primordia has the same origin as gonadal somatic cells (Hatano *et al.*, 1996). Experimental studies using the reaggregate cultures of germ cells with lung somatic cells demonstrated that PGCs obtained from the UGRs at 10.5 dpc already possessed an autonomous program to enter into meiosis (McLaren and Southee, 1997). However, it has remained to be elucidated whether PGCs are induced into meiosis in the UGRs or whether they acquire the capacity to enter into meiosis before reaching there. Our results support the latter possibility, although there remains the possibility that developing UGRs had influenced the migratory PGCs in the mesenteries through a remote mechanism.

Previous studies have shown that male PGCs also enter into meiosis if they have been isolated from UGRs at 10.5 dpc or genital ridges at 11.5 dpc and cultured in reaggregates in the absence of male gonadal environments (McLaren and Southee, 1997). We obtained results similar to this in that

male germ cells at 10.5 or 11.5 dpc expressed Scp3 and began to form axial cores when dissociated and cultured on feeder cells for several days, but those from developing testis at 12.5 or 13.5 dpc showed only weaker signals of Scp3 after the same period of culture. This difference between 11.5- and 12.5-dpc male germ cells *in vitro* supports the previous view that both male and female germ cells are capable of entering meiosis unless influenced by intercellular signals, such as putative meiosis-preventing substances (MPS) (Dolci and De Felici, 1990) supposed to be exerted from the male somatic cells at around 12.5 dpc. The expression of Scp3 in the male germ cells cultured from 12.5 dpc may imply that the dissociation and cultivation procedure decreased the effect of putative MPS and the capacity for meiotic transition was restored to the male germ cells to some extent. The expression of meiosis-specific genes, especially of *Scp3*, in male embryonic gonads *in vivo* also raises the possibility that male fetal germ cells start to prepare for the initial step of meiosis until around 12.5 dpc, and that putative MPS functions after the onset of such preparation.

We seldom observed the progression of meiosis to the pachytene–diplotene stages in this dispersed culture condition, even after maintaining the culture beyond the timing of *in vivo* differentiation to these stages. Since germ cells at these progressed stages were often observed in reaggregated cultures of female genital ridges, further progression of meiosis may require three-dimensional tissue-like structures. Nonetheless, the dispersed culture system developed in this study should serve as a useful tool to analyze the transition from mitosis into meiosis by mouse PGCs.

gp130-Mediated Signaling and Germ-Line Differentiation

gp130-mediated signaling exerts various effects on many types of cells and tissues both *in vitro* and *in vivo*, including hematopoietic cells, neuronal cells, osteopoietic cells, etc. (reviewed in Hilton *et al.*, 1991). As for the effects on pluripotential cells, gp130-mediated signaling maintains the pluripotency and represses the differentiation of ES cells. This signaling is also shown to promote the survival/growth of PGCs and is essential for derivation of pluripotential EG cells from PGCs *in vitro*.

Our observation that gp130-mediated signaling suppressed the expression of Scp3 and inhibited the following formation of axial cores in PGCs could be interpreted as the prevention of meiotic transition *in vitro*. However, it is not clear whether this meiosis-preventing effect has any functional roles *in vivo*. Gene-disrupted mice of the *LIF* and the *LIF receptor* genes did not show any remarkable defects in germ-line development, although there are functional redundancies in the IL-6 cytokine family (Stewart *et al.*, 1992; Ware *et al.*, 1995). Embryos of *gp130* knock-out mice showed a reduced number of PGCs, but differentiation of the remaining germ cells has not been examined, because these mice were embryonic lethal after around the midgestation (Yoshida *et al.*, 1996).

If the observed activity of gp130-mediated signaling has any relation to the *in vivo* regulation of the meiotic transition, one interpretation is that gp130 ligands are involved in functioning of the putative MPS. Alternatively, these ligands may have interfered with the pathway that actually works downstream of the putative MPS. One candidate for such a downstream factor is STAT3 (signal transducer and activator of transcription 3). STAT3 is a transcription factor shown to be necessary and sufficient for the self-renewal of ES cells (Matsuda *et al.*, 1999). It is not unlikely that there exist some pathways by which the differentiation of ES cells and PGCs is regulated in common. It would be of interest to examine the effect of STAT3 or its upstream receptors/ligands on their effects on the growth and/or differentiation of PGCs in a future study.

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